nondermatophytes in onychomycotic nails might influence the outcome of cultures, and the detection rate of nondermatophytes varies according to geographic area,[7] maturity of laboratory techniques, and individual variations among the patients. Nondermatophytes and yeasts occurring with dermatophytes in cultures were previously assumed to be contaminants.[8,9] Recently, however, some authors have emphasized the fact that the frequent occurrence of nondermatophytes plays an important role in the pathogenesis of onychomycosis.[9,10]

Given the degree of uncertainty in conventional culture methods, molecular biological techniques using polymerase chain reaction (PCR) assay provide a rapid, stable and accurate alternative for identifying pathogenic fungi. Molecular biological means of identifying dermatophytes from cultured colonies include arbitrarily primed PCR,[11] PCR-restriction fragment length polymorphism (RFLP),[12] double-round PCR,[13] real-time PCR[14] and PCR-direct sequencing.[15–17] Many attempts have been made with such methods to collect fungal DNA directly from the affected nail samples[5,6,18–20] in order to reduce the false negative results due to failure to grow the fungi in culture media.[5,18,20] However, all of the previous studies were hampered by flaws in the methodology, such as using direct sequencing and RFLP assay for each strain,[6] using primers not specific for each species,[5,18] or not covering common nondermatophytes.[19,20]

In the present study, we analysed the detection rate of medically relevant dermatophytes and nondermatophytes from nail samples of onychomycosis patients by means of a highly sensitive nested PCR assay using newly developed, species-specific primer pairs based on the sequence of the 28S ribosomal RNA gene. Our study accurately indicates the detection rate of dermatophytes and nondermatophytes in onychomycosis nail plates, and will change our views concerning the pathophysiology of onychomycosis based on conventional direct microscopy and fungal culture.

Materials and methods

Standard strains and collection of nail samples

The standard strains used for DNA extraction to ascertain primer specificity are shown in Table 1. All standard strains were identified by ITS1 DNA sequence analysis in addition to conventional identification methods. Fifty Japanese onychomycosis outpatients (36 males and 14 females, 20–82 years old; mean age 57·3 ± 15·1 years) at Tokyo Medical University Hospital were enrolled in the present study after their written informed consent was obtained. Normal-appearing nail samples from 20 healthy Japanese subjects (11 males and nine females, 26–69 years old; mean age 37·9 ± 13·5 years) were

Table 1 Standard strains used in the present study

Species	Strain
Trichophyton rubrum	CN011102601, CN011102609, KTR11, KTR26, T0712, TDF77, T199620
T. mentagrophytes	KTM1, KTM14, KTM18
T. tonsurans	KTT11, KTT16, KTT32
Arthroderma vanbreuseghemii	SM011, SM0110
A. benhamiae	SM103, SM104
Microsporum canis	TIMM1022, CN01072701, T74001, T73015
M. gypseum	T4005, TKAH1210
Epidermophyton floccosum	AZ050223, T1226, T1209
Fusarium oxysporum	T052210, TSY0351
F. solani	KAMI041105, KAMI30212, TSY0403
F. verticillioides	TIMM1294, TIMM1295, TIMM1296, TSY0219
Aspergillus flavus	TIMM2935, JCM2061
A. fumigatus	TIMM2920, JCM10253
A. niger	JCM10254
Scopulariopsis brevicaulis	AF378737, TSY0668
Candida albicans	ATCC10231, TIMM1308
C. dubliniensis	CBS52747
C. krusei	ATCC6258
C. glabrata	ATCC90030
C. guilliermondii	TIMM0257
C. parapsilosis	ATCC22209
C. tropicalis	ATCC750
Pichia anomala	JCM3585, TIMM3826
Saccharomyces cerevisiae	ATCC9763
Geotrichum candidum	TIMM0963
Schizosaccharomyces pombe	L972H
Trichosporon asahii	CN02102217
Cryptococcus neoformans	ATCC900113

© 2009 The Authors
Journal Compilation © 2009 British Association of Dermatologists

also analysed for comparison. Patients who had used an oral antifungal agent in the past or a topical antifungal agent in the past 6 months were excluded from the study. All 50 patients with onychomycosis were ascertained to be positive for fungal elements by board-certified dermatologists using direct microscopy. Small pieces of the nail plates were collected from the distal portion of the affected area using sterile nail clippers.

DNA extraction from fungal cells and clinical nail samples

All fungal strains used to examine primer specificity were grown on Sabouraud dextrose agar (SDA; 1% peptone, 1% glucose and 1·5% agar) at 27 °C for 5 days. DNA from each strain was prepared according to the previously described procedures.[15] Briefly, small amounts of mycelia grown on SDA were placed in lysis buffer [200 mmol L^{-1} Tris–HCl (pH 8·0), 25 mmol L^{-1} ethylenediamine tetraacetic acid, 0·5% sodium dodecyl sulphate and 250 mmol L^{-1} NaCl], and crushed with a conical grinder (Micro multi mixer®; Ieda Chemical, Tokyo, Japan) at a set-up speed for 20–30 s, depending on the species. The samples were then centrifuged at 10 000 *g* for 5 min. The supernatants were extracted once with phenol–chloroform–isoamyl alcohol (25 : 24 : 1, v/v/v), and subsequently extracted once with chloroform. DNA was precipitated with an equal volume of isopropanol at −20 °C for 10 min, washed with 150 μL of 70% ethanol, dried, and suspended in 50 μL of ultrapure water (Milli-Q Synthesis A10; Millipore, Bedford, MA, U.S.A.). Aliquots of 1 μL of the resultant solution were used as templates for the PCR.

Nail clippings collected from patients with onychomycosis were crushed by using a multibead shocker (Yasui Kikai, Osaka, Japan) with 3 g of metal corn at 1800 rpm for 20 s. The ground nail samples were mixed with lysis buffer, and DNA was extracted by the aforementioned method and suspended in 50 μL of ultrapure water. Aliquots of 25 μL of the resultant solutions were used as templates for the first PCR of clinical samples.

Specific oligonucleotide

Fungus universal primers (FUP) were prepared to detect 28S ribosomal RNA gene sequences from all medically relevant fungi. The FUP 28SF1 primer was newly designed based on the sequences of *C. albicans*, X70659; *Cryptococcus neoformans*, C14068; *Mucor*, M26190; while the FUP 635 primer was identical with the sequence reported by Guého *et al.*[21] (Table 2). In addition, three dermatophyte-specific PCR primer pairs, as well as five nondermatophyte-specific primer pairs, and two *Candida*-specific primer pairs based on the 28S rRNA gene were newly designed (Table 2). Dermatophyte universal primers (DPUP) were prepared in order to detect all of the pathogenic dermatophytes including *T. rubrum* and *T. mentagrophytes*. All these primers were designed from the inner region of FUP, and were therefore used as the primers for nested PCR. Oligonucleotides in this study were designed based

Table 2 Primer pairs used in the present study

Target	Primer name	Sequence (5′>3′)	PCR products size (bp)
Fungus	FUP[a] 28SF1	AAGCATATCAATAAGCGGAGG	600–650
	FUP[a] 635	GGTCCGTGTTTCAAGACGG	
Dermatophyte	DPUP[b] F1	AGTAGAGTGATCGAAAGGTT	273–275
	DPUP[b] R1	ATTACGCCAGCATCCGAG	
T. rubrum	TRUB F1	CGTCGCCCGTGCACTG	137
	TRUB R1	GAGCGCGTTCCTCAGTCT	
T. mentagrophytes	TMENT F1	GTGCTCGTCGCCCGTGT	102
	TMENT R1	GGCTATAAGACGTCCCG	
Aspergillus spp.	ASP F1	GCATTCGTGCCGGTGTACTT	170
	ASP R1	TTACGACCATTATGCCAGCG	
S. brevicaulis	SCOP F1	CGTCGGATCAACCGTCGCTT	168
	SCOP R1	ACGCCAGCATCCTTGCATAC	
F. solani	FSOL F1	GTAGAGGATGCTTTTGGTGA	291
	FSOL R1	CAAGCCCAAGTCTGGTCAC	
F. oxysporum	FOXY F1	ATCTCTGTAAAGTTCCTTCA	335
	FOXY R1	CCCAGGGTATTACACGGT	
F. verticillioides	FVER F1	ATCTCTGTAAAGTTCCTTCG	296
	FVER R1	AGAGCCACATTCCCGAAGT	
C. albicans	CALB F1	TGAAATCTGGCGTCTTTGGC	445
	CALB R1	GACGCTATAACACACAGCAG	
C. tropicalis	CTRO F1	TTTGAAATCTGGCTCTTTCAGA	419
	CTRO R1	GCCACATTCCAACGCAATTC	

[a]Fungus universal primers; [b]dermatophyte universal primers.

© 2009 The Authors

on the sequences of the 28S rRNA gene in the DDBJ/EMBL/GenBank databases (Accession numbers: *T. rubrum*: AF378734, AY176744, AY213629; *T. mentagrophytes*: AF378739, AF378740, AF378738; *Trichophyton tonsurans*: AF448547; *Arthroderma benhamiae*: AY315660; *Trichophyton violaceum*: AB084194246, AF506035, AJ232189; *Epidermophyton floccosum*: AY176734, AF378736; *Microsporum canis*: AF448550, AY176735; *Aspergillus niger*: U28812, U28814, U28815, U28816, U28817; *Aspergillus flavus*: U28900, U15487, U28889, AB105349; *Scopulariopsis brevicaulis*: AF378737; *Fusarium solani*: AF130382, AF130383, AY097316, AY097317, AY097318; *F. oxysporum*: AB084299, AF130373, U34537, U34542; *F. verticillioides*: U34528, U68130, AF130385, AF130384; *Acremonium spp.*: AF130386; *Acremonium tubakii*: AY305033; *Histoplasma capsulatum*: U26906, U26907; *Coccidioides immitis*: AY176713, AB040702, AY176713, AB040702). The base sequences specific to each primer were analysed with GENETYX-MAC Version 10 (Software Development Co. Ltd, Tokyo, Japan) and the oligonucleotide primers were manufactured by Amersham-Pharmacia Biotech Co. Ltd (Tokyo, Japan). The size of the amplified products was estimated to be 102–650 bp (Table 2).

Polymerase chain reaction (PCR) amplification and detection of PCR products

Each PCR assay contained 10 μL of 10 × reaction buffer, 100 $\mu mol\ L^{-1}$ each of dATP, dCTP, dGTP and dTTP (Amersham Biosciences Corp., Piscataway, NJ, U.S.A.), 2·5 U of Taq polymerase, 30 pmol of each primer, and 1 μL of the DNA template solution. Ultrapure water was added to bring the volume to 100 μL. Each mixture was heated to 94 °C for 5 min, and PCR was performed for 25 cycles under the following conditions: 94 °C for 1 min, 60 °C for 15 s and 72 °C for 15 s. Thermal cycling was terminated by polymerization at 72 °C for 10 min. The PCR program for *F. oxysporum* (Foxy F1 and Foxy R1) was modified as follows: 94 °C for 1 min, 55 °C for 15 s and 72 °C for 15 s. Aliquots of 5 μL of the amplified products were subjected to electrophoresis on a 1·2% agarose gel and visualized by staining with ethidium bromide under UV irradiation. All the amplified PCR products were directly sequenced by DNA Sequencing Kit (Applied Biosystems) and an automatic sequencer (Genetic Analyzer 310, Applied Biosystems) according to the manufacturer's instructions. The sequences were compared with the 28S sequence of each fungus using GENETYX-MAC 10 (Software Development Co. Ltd) to confirm a 100% match.

The first PCR with 25 μL of DNA template solution from nail samples was performed using FUP for 30 cycles under the following conditions: 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1·5 min. When the products of the first PCR were visualized, the sample solution was diluted 100 times with distilled water, and aliquots of 1 μL of the resultant solution were then used in the nested PCR. Meanwhile, aliquots of 1 μL of undiluted solution from the first PCR were used as templates for the nested PCR when the products of the first PCR were not visualized.

Results

Specificity and sensitivity of the primer pairs

The specificity of each primer pair (Table 2) was examined by using DNA templates prepared from fungal cells in culture. The products of 0·1–0·4 kb were amplified by PCR from each species. No amplification product was observed without a DNA template, or by using primer pairs from other species. All fungal DNAs from species shown in Table 1 were amplified successfully with FUP, and corresponding dermatophyte and nondermatophyte DNAs were detected by using DPUP and species-specific primers, respectively. FUP for 28S ribosomal RNA gene were used to amplify DNAs from all medically relevant fungi except *Coccidioides immitis* (data not shown). DPUP did not amplify the DNAs collected from nondermatophytes or yeasts.

The sensitivity of PCR was assessed using FUP and *Aspergillus* spp.-specific primers (ASP F1, ASP R1) under the conditions described in Materials and methods. In conjunction with ethidium bromide staining, the FUP were able to detect 100 copies of *A. flavus* plasmid DNA, while the *Aspergillus* spp.-specific primers were able to detect 1000 copies of *A. flavus* plasmid DNA (data not shown).

All of the amplified PCR products were directly sequenced and confirmed to be identical with the reported sequences of each fungal 28S ribosomal RNA gene in the DDBJ/EMBL/GenBank databases.

Results of the first polymerase chain reaction (PCR) and subsequent nested PCR using clinical onychomycosis nail samples

Fifty clinical nail samples, determined to be positive for onychomycosis by direct microscopy, were collected from the affected great toenail of 50 Japanese subjects. As shown in Table 3, the first PCR and subsequent nested PCR were performed using newly designed dermatophyte and nondermatophyte specific primer pairs listed in Table 2. The first PCR showed 35 positive samples, and the nested PCR showed 47 positive samples. Three samples failed to amplify any fungal DNA in either of the assays. The total fungal detection rate using this method was 94% (47 of 50 cases). Nested PCR products from clinical nail samples were directly sequenced and found to match exactly the reported 28S rRNA gene sequences of each species. Nail samples collected from 20 healthy subjects were investigated under PCR conditions identical with those mentioned previously. Repeated experimental procedures indicated that one of 20 samples was positive at the nested PCR stage for *Aspergillus* spp., suggesting that the nail plate was contaminated with the organism.

The fungal detection rate for each of the nested PCR-positive 47 cases was as follows: dermatophytes: 39 cases (83·0%): *T. rubrum*, 35 cases (74·5%) and *T. mentagrophytes*, eight cases (17·0%); nondermatophytes: *Aspergillus* spp.: 12 cases (25·5%), *F. solani*: five cases (10·6%), *F. verticillioides*: two cases

© 2009 The Authors
Journal Compilation © 2009 British Association of Dermatologists

Table 3 Results of nested PCR from onychomycotic nail samples

	Sample no.																								
Primer sets specific for	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
FUP[a] (first PCR)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
S. brevicaulis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aspergillus spp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F. solani	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F. oxysporum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F. verticillioides	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
T. rubrum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T. mentagrophytes	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
DPUP[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. albicans	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
C. tropicalis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

	Sample no.																								
Primer sets specific for	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
FUP[a] (first PCR)	+	+	−	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
S. brevicaulis	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aspergillus spp.	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
F. solani	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−
F. oxysporum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
F. verticillioides	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
T. rubrum	−	−	−	+	−	−	−	−	−	−	+	+	+	+	−	+	−	+	+	−	+	+	−	−	−
T. mentagrophytes	+	+	+	−	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	+	+	−	−	−
DPUP[b]	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	−	+	+	−	+	+	−	−	−
C. albicans	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
C. tropicalis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a]Fungus universal primers; [b]dermatophyte universal primers.

(4·3%), *F. oxysporum*: one case (2·1%), *S. brevicaulis*: one case (2·1%); *C. albicans*: four cases (8·5%), *C. tropicalis*: zero cases (0%). *T. rubrum* was the first, and *T. mentagrophytes* the second, most frequently observed dermatophyte in the affected nail plates. Occurrence of onychomycosis due to other dermatophytes was estimated to be very low as dermatophyte-positive and *T. rubrum*- and *T. mentagrophytes*-negative cases were not observed in the present study. Unexpectedly, nondermatophytes were seen in 18 cases (38·3%), dermatophytes and nondermatophytes in 10 cases (21·3%) and nondermatophytes alone in eight cases (17·0%). *Aspergillus* spp. alone was observed in five cases (10·6%) and *F. oxysporum* alone in one case (2·1%). *C. albicans* was not observed as the sole fungal agent in any of the samples.

Discussion

Recent advances in molecular biological techniques using PCR assays have dramatically increased the sensitivity and specificity of methods for identifying pathogenic microorganisms, and have been used to good effect in identifying pathogenic fungi in the affected nail samples of patients with onychomycosis as well.[5,6,18–20] Even in cases where the quantity of nail plate samples is limited, amplification of pure fungal DNA is feasible with the newly developed, highly sensitive methods as nail plates contain minimal quantities of host-derived DNAs. Double-round PCR[13,20] and nested-PCR, used in the present study, are highly sensitive and specific means of detecting trace amounts of fungal DNAs from nail plate samples.

The identification rate of 94% obtained in our study is extremely high compared with that of the conventional cultures.[4–6] Only three of 50 samples from the microscopy-positive nail plates failed to amplify any fungal DNA, suggesting that the organisms were not contained in the collected samples, or that the organisms observed by microscopy were not viable by virtue of having denatured DNA or insufficient quantities of intact DNA. In view of these facts, we can confidently assert that the identification of fungal species is possible in almost all of the microscopy-positive samples, and that failure of fungal growth in any of the microscopy-positive samples was due to technical imperfection in the culture or to nonviable organisms with intact DNAs, rather than to the absence of pathogenic fungi in the affected nail plates. Fungal culture was not performed in the present study because larger quantities of nail sample were

© 2009 The Authors
Journal Compilation © 2009 British Association of Dermatologists

required for DNA extraction, and direct microscopy was reported to be a more sensitive diagnostic tool than fungal culture.[4,5]

Toenail onychomycosis is caused by dermatophytes, nondermatophyte moulds, yeasts, or a combination of these agents. When we restricted molecular identification to the data on dermatophytes, our nested-PCR results indicated a 70·0% positive rate for *T. rubrum* (35 of 50 cases) and a 16·0% positive rate for *T. mentagrophytes* (eight of 50 cases) from the microscopy-positive nail samples. Although the detection rate of *T. rubrum* from the nail samples by molecular biological means varies depending on the specific method employed, sample sources and clinical manifestations,[5,20] the results are greatly superior to those obtainable from conventional cultures (e.g. 39% positive rate for *T. rubrum* from the microscopy-positive nail samples[4]). In our analysis of dermatophytes, we prepared primer pairs only for dermatophyte universal, *T. rubrum* and *T. mentagrophytes*. However, dermatophytic onychomycosis caused by other pathogenic dermatophytes was not observed, corroborating previous reports that it is relatively rare.[2,22–24]

In the present study, nondermatophytes were detected in as many as 38% of the tested cases, including cases of mixed infection involving dermatophytes. This frequency rate was much higher than in the previously reported surveys in which prevalence rates of nondermatophytic onychomycosis ranged from 1·45% to 17·6%.[25] Recently, mixed infections in onychomycosis cases have been detected with increasing regularity,[9] thanks to the application of new techniques in immunohistochemistry, flow cytometry[26] and molecular biology.[6] By preparing species-specific primer pairs for nondermatophytes and yeasts, we succeeded in obtaining accurate detection rates for each species in the affected nail samples.

For fungal detection, nondermatophyte mould and yeast species were selected on the basis of identification frequencies in onychomycosis cases reported in previous studies.[2,22–24] *Scytalidium* spp. were excluded from the assay by reason of their geographic distribution. *Acremonium* spp. were strong candidates for primer designing, but because appropriate primer pairs could not be designed at the inner site of the FUP, they were excluded from our study. Although the possibility of mixed infection with *Acremonium* spp. cannot be ruled out in our study, primary infection by the species could be definitely ruled out, given that FUP-positive and nested PCR-negative samples were not observed. The result of sequencing the first PCR products from some of the samples also failed to match the DNA sequence for *Acremonium* spp. *Candida tropicalis* was not detected in the present study, although researchers have previously documented the occurrence of *C. tropicalis* onychomycosis.[27,28] The prevalence rate of *Candida* spp. in the great toenails might be low.[24]

Summerbell[29] has suggested the following categories for nondermatophyte moulds isolated from onychomycosis samples: (i) contaminant; (ii) normal mammalian surface commensal; (iii) transient saprobic colonizer; (iv) persistent secondary colonizer; (v) successional invader; (vi) primary invader. It should be noted that the contamination that can occur during sample collection, DNA extraction and PCR procedures may produce misleading results due to the extreme sensitivity of nested PCR assays.[30] In any event, the purpose of our study is to indicate the detection rate of various fungi involved in onychomycosis using a sensitive and specific method, and not to determine their pathogenicity. A single PCR assay performed prior to treatment is not sufficient to determine the pathogenicity of a detected nondermatophyte; positive results in repeated PCRs with successively collected samples and a positive culture are necessary for the diagnosis of nondermatophyte onychomycosis.

Aspergillus spp. and *Fusarium* spp. sometimes occur as contaminants, colonizers, or the sole infectious organism in onychomycosis.[25,31,32] Our study also demonstrated that *Aspergillus* spp.-derived fungal DNAs occur in one of 20 normal-appearing nails. Dermatophytes were isolated from normal-appearing nails at a rate of 4·7% in cultures[4] and 3·7% by PCR.[5] Although we do not have definitive evidence, nondermatophytes in some of the mixed infection cases might not be contaminants or colonizers. Mixed infections by *T. rubrum* and *T. mentagrophytes* were observed in four cases in our study. Only nondermatophytes were observed in eight cases (17·0%), of which five cases involved *Aspergillus* spp. alone and one case, *F. oxysporum* alone. The first PCR was positive in four of eight cases, suggesting the presence of a large population of organisms in the nail samples. A negative result for dermatophytes by nested PCR indicates the absence of any dermatophytic organisms in the nail sample, suggesting that the detected nondermatophyte was very likely to be the cause of the onychomycosis.

Management of nondermatophyte onychomycosis is not always successful. Systemic itraconazole or terbinafine might be the first line of treatment for nondermatophytic onychomycosis as well as dermatophytic onychomycosis. Nonetheless, although terbinafine has higher *in vitro* antifungal activity compared with itraconazole against *Scopulariopsis* and *Acremonium* spp., and, in contrast, itraconazole has lower minimum inhibitory concentration (MIC) values than terbinafine against *Fusarium*, *Aspergillus* and *Candida* spp.,[22] the clinical relevance of the MIC of these oral antifungals remains uncertain. In fact, in the present study, all of the patients suffering from onychomycosis due to *Aspergillus* spp. alone were cured by means of pulse therapy with 400 mg day^{-1} itraconazole (data not shown).

Our study clearly demonstrated that the frequency of nondermatophytes and yeasts detected in onychomycosis is much higher than we had expected. The highly sensitive and specific molecular detection method employed is extremely useful for cases of nondermatophytic onychomycosis in which the causative organisms cannot be isolated by conventional culture, and for determining the choice of appropriate antifungals in intractable cases. In the present study, we saw little difference between dermatophyte and nondermatophyte onychomycosis in both direct microscopic observation and macroscopic observation of the affected nails (data not shown). Collecting a

© 2009 The Authors
Journal Compilation © 2009 British Association of Dermatologists

larger number of samples and successively sampling for PCR and culture after commencement of antifungal therapy are required to better understand the pathophysiology of nondermatophyte onychomycosis and to obtain any data correlating with the clinical manifestations.

Acknowledgments

This work was supported by the 'High-Tech Research Centre' Project for private universities, a matching fund subsidy from the MEXT (Ministry of Education, Culture, Sports, Science and Technology of JAPAN, 2003-2007) (R.T.). We thank our colleagues in our department for their clinical contributions and Mr Wataru Suzuki for his valuable suggestions.

References

1 Elewski BE. Onychomycosis: pathogenesis, diagnosis, and management. *Clin Microbiol Rev* 1998; **11**:415–19.

2 Gupta AK, Jain HC, Lynde CW *et al.* Prevalence and epidemiology of unsuspected onychomycosis in patients visiting dermatologists' offices in Ontario, Canada—a multicenter survey of 2001 patients. *Int J Dermatol* 1997; **36**:783–7.

3 Monod M, Baudraz-Rosselet F, Ramelet AA *et al.* Direct mycological examination in dermatology: a comparison of different methods. *Dermatologica* 1989; **179**:183–6.

4 Davies RR. Mycological tests and onychomycosis. *J Clin Pathol* 1968; **21**:729–30.

5 Kardjeva V, Summerbell R, Kantardjiev T *et al.* Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. *J Clin Microbiol* 2006; **4**:1419–27.

6 Monod M, Bontems O, Zaugg C *et al.* Fast and reliable PCR/sequencing/RFLP assay for identification of fungi in onychomycosis. *J Med Microbiol* 2006; **55**:1211–16.

7 De Doncker P, Scher RK, Baran RL *et al.* Itraconazole therapy is effective for pedal onychomycosis caused by some nondermatophyte molds and mixed infection with dermatophytes and molds: a multicenter study with 36 patients. *J Am Acad Dermatol* 1997; **36**:173–7.

8 Gianni C, Cerri A, Crosti C. Non-dermatophytic onychomycosis: an underestimated entity? A study of 51 cases. *Mycoses* 2000; **43**:29–33.

9 Vander Straten MR, Balkis MM, Ghannoum MA. The role of nondermatophyte molds in onychomycosis: diagnosis and treatment. *Dermatol Ther* 2002; **15**:89–98.

10 Gupta AK, Cooper EA, MacDonald P, Summerbell RC. Utility of inoculum counting (Walshe and English criteria) in clinical diagnosis of onychomycosis caused by nondermatophytic filamentous fungi. *J Clin Microbiol* 2001; **39**:2115–21.

11 Liu D, Coloe S, Baird R *et al.* Molecular determination of dermatophyte fungi using the arbitrarily primed polymerase chain reaction. *Br J Dermatol* 1997; **137**:351–5.

12 Kamiya A, Kikuchi A, Tomita Y *et al.* PCR and PCR-RFLP techniques targeting the DNA topoisomerase 2 gene for rapid clinical diagnosis of the etiologic agent of dermatophytosis. *J Dermatol Sci* 2004; **34**:35–48.

13 Turin L, Riva F, Galbiati G *et al.* Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. *Eur J Clin Invest* 2000; **30**:511–18.

14 Gutzmer R, Mommert S, Küttler U *et al.* Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. *J Med Microbiol* 2004; **12** (Pt 12):1207–14.

15 Makimura K, Murayama S, Yamaguchi H. Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Med Microbiol* 1994; **40**:358–64.

16 Makimura K, Tamura Y, Mochizuki T *et al.* Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. *J Clin Microbiol* 1999; **37**:920–4.

17 Ninet B, Jan I, Bontems O *et al.* Molecular identification of *Fusarium* species in onychomycoses. *Dermatology* 2005; **10**:21–5.

18 Arabatzis M, Bruijnesteijn van Coppenraet LE, Kuijper EJ *et al.* Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme. *Br J Dermatol* 2007; **157**:681–9.

19 Baek SC, Chae HJ, Houh D *et al.* Detection and differentiation of causative fungi of onychomycosis using PCR amplification and restriction enzyme analysis. *Int J Dermatol* 1998; **37**:682–6.

20 Gupta AK, Zaman M, Singh J. Fast and sensitive detection of *Trichophyton rubrum* DNA from the nail samples of patients with onychomycosis by a double-round polymerase chain reaction-based assay. *Br J Dermatol* 2007; **157**:698–703.

21 Guého E, Kurtzman CP, Peterson SW. Evolutionary affinities of heterobasidiomycetous yeasts estimated from 18S and 25S ribosomal RNA sequence divergence. *Syst Appl Microbiol* 1989; **12**:230–6.

22 Ghannoum MA, Hajjeh RA, Scher R *et al.* A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns. *J Am Acad Dermatol* 2000; **43**:641–8.

23 Gupta AK, Konnukov N, MacDonald P *et al.* Prevalence and epidemiology of toenail onychomycosis in diabetic subjects: a multicentre survey. *Br J Dermatol* 1998; **139**:665–7.

24 Midgley G, Moore MK, Cook JC *et al.* Mycology of nail disorders. *J Am Acad Dermatol* 1994; **31**:S68–74.

25 Tosti A, Piraccini BM, Lorenzi S. Onychomycosis caused by nondermatophytic molds: clinical features and response to treatment of 59 cases. *J Am Acad Dermatol* 2000; **42**:217–24.

26 Piérard GE, Arrese JE, Doncker PD *et al.* Present and potential diagnostic techniques in onychomycosis. *J Am Acad Dermatol* 1996; **34**:273–7.

27 Chun DK, Lee UH, Park HS. Onychomycosis in a premature infant caused by *Candida tropicalis*. *J Eur Acad Dermatol Venereol* 2004; **18**:617–18.

28 Ellabib MS, Agaj M, Khalifa Z *et al.* Yeasts of genus *Candida* are the dominant cause of onychomycosis in Libyan women but not men: results of a 2-year surveillance study. *Br J Dermatol* 2002; **146**:1038–41.

29 Summerbell RC. Epidemiology and ecology of onychomycosis. *Dermatology* 1997; **194** (Suppl. 1):32–6.

30 Loeffler J, Hebart H, Bialek R *et al.* Contaminations occurring in fungal PCR assays. *J Clin Microbiol* 1999; **37**:1200–2.

31 Baran R, Tosti A, Piraccini BM. Uncommon clinical patterns of *Fusarium* nail infection: report of three cases. *Br J Dermatol* 1997; **136**:424–7.

32 DiSalvo AF, Fickling AM. A case of nondermatophytic toe onychomycosis caused by *Fusarium oxysporum*. *Arch Dermatol* 1980; **116**:699–700.

© 2009 The Authors

Journal Compilation © 2009 British Association of Dermatologists

Disseminated Aspergillosis Following Resolution of *Pneumocystis* Pneumonia with Sustained Elevation of Beta-Glucan in an Intensive Care Unit: a Case Report

T. Saito, N. Shime, K. Itoh, N. Fujita, Y. Saito, M. Shinozaki, K. Shibuya, K. Makimura, S. Hashimoto

Abstract
Invasive aspergillosis is a major cause of morbidity and mortality in immunocompromised patients receiving intensive care. The double-sandwich ELISA for galactomannan is reported to have a high sensitivity (96.5%) for the detection of invasive aspergillosis when a cut-off value of 0.8 ng/ml is used. However, we have experienced a case of lethal disseminated aspergillosis in a patient that presented with a negative galactomannan (GM) test and persistent elevation of β-D glucan (BG) levels. A 63-year-old female was admitted to our Intensive Care Unit (ICU) in acute respiratory failure and elevated BG. She had been receiving medication for Goodpasture syndrome based on anti-glomerular basement membrane antibodies and myeloperoxidase–antineutrophil cytoplasmic antibodies for 9 months and was receiving long-term prednisolone therapy (20 mg/day). On admission, her trachea was immediately intubated, and a PCR analysis of the bronchoalveolar lavage sample revealed *Pneumocystis jiroveci*. Trimethoprimsulfamethoxazole therapy was started for *Pneumocystis* pneumonia. The levels of BG remained elevated (> 100 pg/ml) during the treatment period despite the clinical resolution of *Pneumocystis* pneumonia, raising concerns of another complicated invasive fungal disease; consequently, fosfluconazole was administered empirically. The serum BG levels, however, did not decrease. Blood cultures did not detect a fungal infection. Serum GM levels measured by a double-sandwich ELISA on the 6th, 11th, and 24th days in the ICU were negative (< 0.2 ng/ml). The patient ultimately died of multiple organ failure on the 45th ICU day. Postmortem examination revealed a disseminated fungal infection with aggressive vascular invasion of the lungs, heart, and brain. In situ hybridization with a 568-bp probe of the alkaline proteinase sequence of *Aspergillus fumigatus* showed specific positive staining within the fungus present in the infected lung tissue, revealing that this patient may have had a systemic infection by *A. fumigatus* or *A. flavus*. This is a case of serum GM-negative disseminated aspergillosis pathologically proven by autopsy. Persistent elevated BG levels (> 100 pg/ml) refractory to trimethoprim–sulfamethoxazole and fosfluconazole may suggest possible *Aspergillus* infection and should prompt the initiation of empiric anti-aspergillosis therapies in patients at risk for fungal infection.

Infection 2009; 37: 547–550
DOI 10.1007/s15010-009-8108-5

Introduction

Invasive aspergillosis (IA) is a major cause of morbidity and mortality in immunocompromised patients receiving intensive care. The lung is the main portal of entry and is affected in 80–90% of cases of IA [1]. Disseminated aspergillosis (DA) is the most fulminant presentation of IA, diffusely involving multiple organ systems. The case-fatality rate of DA is as high as 88.1% [2], which strongly indicates the need for prevention or earlier diagnosis and treatment. The prevalence of IA is increasing in Japan and the USA [3, 4], but autopsy studies have revealed that the diagnosis of IA is commonly missed in Intensive Care Units (ICUs) [5].

A definition of invasive fungal infection has been developed [6, 7], but the early diagnosis of IA is still challenging. Good clinical culture samples are frequently unavailable, so clinicians have recently been utilizing several adjunctive diagnostic methods, such as the galac-

T. Saito *(corresponding author)*, **Y. Saito**
Division of Thoracic Surgery, Dept. of Thoracic and Cardiovascular Surgery, Kansai Medical University Hirakata Hospital, 2-3-1 Shin-machi, Hirakata, 573-1191, Japan; Phone: (+81/72) 804-0101, Fax: -2865, e-mail: saitotom@hirakata.kmu.ac.jp
N. Shime, S. Hashimoto
Dept. of Anesthesiology and Intensive Care, Kyoto Prefectural University of Medicine, Kyoto, Japan
K. Itoh
Dept. of Pathology and Applied Neurobiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan
N. Fujita
Dept. of Molecular Genetics and Laboratory Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan
M. Shinozaki, K. Shibuya
Dept. of Pathology, Toho University School of Medicine, Tokyo, Japan
K. Makimura
Dept. of Molecular Biology and Gene Diagnosis, Institute of Medical Mycology, Teikyo University, Tokyo, Japan

Received: March 15, 2008 · Revision accepted: March 2, 2009
Published online: September 2, 2009

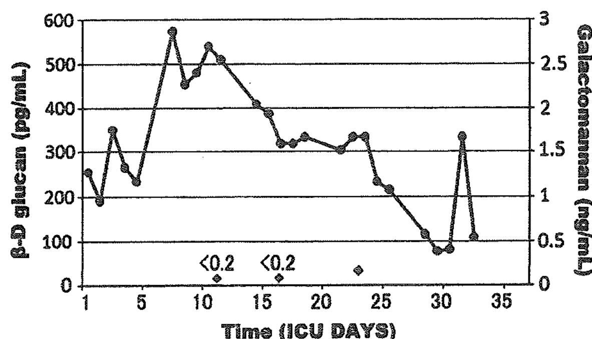


Figure 1. Kinetics of the (1 → 3)-β-D-glucan (BG; filled circles) and galactomannan (GM; diamonds) assays in this case. The GM levels were persistently negative (<0.2 ng/ml), whereas the BG concentration showed a sustained elevation (> 100 pg/ml) and resurged on the 31st ICU day.

tomannan (GM) antigen detection test or the (1 → 3)-β-D-glucan (BG) assay. The double-sandwich ELISA for the detection of GM is reported to have high sensitivity (96.5%) and high specificity (97.3%) for IA when a cut-off value of 0.8 ng/ml is used [8]. However, we have recently experienced a case of lethal DA in which the patient presented with a negative GM test and persistent elevated BG levels.

Case Presentation

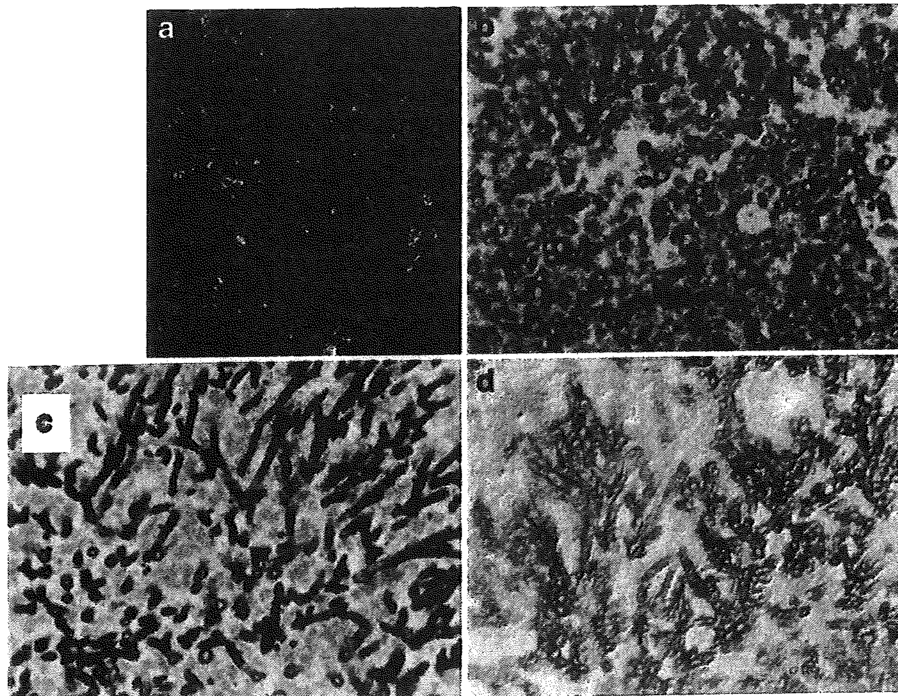
A 63-year-old female was admitted to our ICU in acute respiratory failure with a decreased a PaO_2/FiO_2 (P/F) ratio < 200 and elevated BG (β-D glucan test Wako; Wako Pure Chemical Industries, Tokyo, Japan) and C-reactive protein levels. She had been receiving medication for Goodpasture syndrome with anti-glomerular basement membrane (GBM) antibodies and myeloperoxidase-anti-neutrophil cytoplasmic antibodies (MPO-ANCA) for 9 months, long-term prednisolone therapy (20 mg/day), and intravenous immunoglobulin infusion or intermittent hemodialysis. During the treatment period she had alveolar hemorrhage, cytomegalovirus (CMV) infection, duodenal ulcer, and crusted scabies.

On admission, her trachea was immediately intubated and bronchoalveolar lavage (BAL) was performed. A PCR analysis of the BAL sample revealed *Pneumocystis jiroveci*, and trimethoprim–sulfamethoxazole was started for *Pneumocystis* pneumonia (PCP). Her P/F ratio improved to 300 on the tenth day in the ICU, and the *P. jiroveci* became undetectable by PCR on the 23rd ICU day. The levels of BG, however, showed elevated (> 100 pg/ml) during the treatment period, despite clinical resolution of the PCP (Figure 1).

The high BG levels raised concerns of another complicated invasive fungal disease and, therefore, fosfluconazole was administered empirically. The serum BG levels, however, did not decrease. Blood cultures did not detect a fungal infection. Serum GM levels measured by double-sandwich ELISA (Platelia Aspergillus; Bio-Rad, Marnes La Coquette, France) on the 6th, 11th, and 24th ICU days were negative (≤ 0.2 ng/ml), and there was no prolonged neutropenia (< $500/mm^3$ for > 10 days) during the ICU stay.

The patient subsequently developed *Pseudomonas aeruginosa* bacteremia and a CMV infection relapse, necessitating long-term antimicrobial therapy. She died of multiple organ failure on the 45th ICU day. Postmortem examination revealed a disseminated fungal infection with aggressive vascular invasion and abscess formation in the heart, brain, and lungs (Figigure 2a–c). In situ hybridization with a 568-bp probe of the alkaline

Figure 2. a) Macroscopic picture and microphotographs of lung tissue from the patient described here, b) an abscess formation on the surface of left upper lobe, c) hematoxylin-eosin staining, d) Grocott stain shows proliferating fungi with angioinvasion; DNA in situ hybridization with the alp-568 probe demonstrates specific signal within the same area.



proteinase sequence of *A. fumigatus* showed a specific positive staining within the fungus present in the infected lung tissue (Figure 2d), indicating that this patient may have had a systemic infection by *A. fumigatus* or *A. flavus*. Crescentic glomerulonephritis and pulmonary capillaritis was also found. *Pneumocystis jiroveci* was not found in the lungs.

Discussion

Here we report a rare case of autopsy-proven DA with false-negative GM test results. It has been reported that *A. fumigatus* and *A. flavus* can be detected in infected tissue by in situ hybridization with an alp-568-bp probe [9]. Our patient suffered from Goodpasture syndrome with MPO-ANCA and anti-GBM antibody, and such individuals are reportedly at high risk of severe infection [10].

GM is a polysaccharide cell-wall component that is released by *Aspergillus* spp during mycelial growth. The sandwich ELISA test recognizes (1 → 5)-β-D-galactofuranose side chains of GM with the EB-A2 monoclonal antibody [11]. The GM test has a high specificity and a wide range of sensitivity (30–100%) [12]. The major reasons for false-negative results are high cut-off values and exposure to antifungal agents [13]. Sensitivity can be improved to 96.5% when a cut-off of 0.8 ng/ml is used [8], but the serum GM levels were < 0.2 ng/ml in our case. Although antifungal drugs, such as amphotericin B, decrease the release of galactofuran antigens by reducing fungal angioinvasion in neutropenic rabbits [14], our patient ultimately developed DA with aggressive vascular invasion. Consequently, the administration of fosfluconazole did not have an effect on the expression of GM antigenemia.

The persistently negative GM levels in this case may have been due to the presence of *Aspergillus* spp antibody, which can interfere with the performance of the sandwich ELISA [9], although this is an unproven speculation. Another possibility is that this *Aspergillus* may secrete a GM antigen with only one galactofuranose epitope, which cannot be detected by the sandwich ELISA with EB-A2 [10].

Another interesting feature in this case is the dissociation of the two adjunctive fungal parameters, GM and BG. The BG, a polysaccharide component of fungal cell walls, is an adjunctive parameter suggesting possible deep mycosis, but it is nonspecific with respect to fungal species [15, 16]. BG is also a reliable marker for the diagnosis of *Pneumocystis* pneumonia [17]. It is also known that serum BG levels are elevated in patients undergoing hemodialysis with modified regenerated cellulose membranes, but we used synthetic polysulfone membranes, which do not affect the BG levels [18]. Retrospectively, the resurgence of BG on the 31st ICU day may have indicated the systemic *Aspergillus* infection. Thus, this case suggests that if the BG level does not change despite clinical and pathological improvement of *Pneumocystis* pneumonia, clinicians should consider the possibility of complicated fungal infections. Given the ineffectiveness of empirically administered fosfluconazole, we should have suspected invasive infection by fosfluconazole-resistant fungi, such as *Aspergillus* spp. Additional empiric treatment with, for example, polyenes, mold active azoles (such as voriconazole [19, 20]), or echinocandins should have been considered, especially given the greater risk of fungal infection presented by this patient due to persistent immunosuppression from long-term steroid and antimicrobial therapy.

Conclusion

We report a case of serum GM-negative disseminated aspergillosis pathologically proven by autopsy. Persistent elevation in the BG levels (>100 pg/ml) refractory to trimethoprim–sulfamethoxazole and fosfluconazole drug therapy may suggest possible *Aspergillus* infection and should prompt the clinician to initiate empiric anti-aspergillosis therapies in patients at risk for fungal infections.

Acknowledgments

We would express our gratitude to Dr. Tadaaki Hiruki for his English editing of this manuscript.

References

1. Denning DW: Invasive aspergillosis. Clin Infect Dis 1998; 26: 781–805.
2. Lin SJ, Schranz J, Teutsch SM: Aspergillosis case-fatality rate: systemic review of the literature. Clin Infect Dis 2001; 32: 358–366.
3. Yamazaki T, Kume H, Murase S, Yamashita E, Arisawa M: Epidemiology of visceral mycoses: analysis of data in annual of the pathological autopsy cases in Japan. J Clin Microbiol 1999; 37: 1732–1738.
4. Dasbach EJ, Davis GM, Teusch SM: Burden of aspergillosis-related hospitalization in the United States. Clin Infect Dis 2000; 31: 1524–1528.
5. Thomas CM, Neil SY: The relationship of pre mortem diagnosis and post mortem findings in a surgical intensive care unit. Crit Care Med 1999; 27: 299–303.
6. Ascioglu S, Rex JH, de Pauw BE, Bennett JE, Bille J, Crokaert F, et al. Defining opportunistic invasive fungal infection in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 2002; 34: 7–14.
7. de Pauw BE, Patterson TF: Should the consensus guidelines' specific criteria for diagnosis of invasive fungal infection be changed? Clin Infect Dis 2005; 41: S377–S380.
8. Maertens J, Theunissen K, Verbeken E, Lagrou K, Verhaegen J, et al. Prospective clinical evaluation of lower cut-offs for galactomannan detection in adult neutropenic cancer patients and haematological stem cell transplant recipients. Br J Haematol 2004; 126: 852–860.
9. Hanazawa R, Yamagata S, Yamaguchi H: In situ detection of Aspergillus fumuigatus. J Med Microbiol 2000; 49: 285–290.
10. Levy JB, Hammad T, Coulthart A, Dougan T, Pusey CD: Clinical features and outcome of patients with both ANCA and anti-GBM antibodies. Kidney Int 2004; 66: 1535–1540.

11. Mennink-Kersten MA, Donnelly JP, Verweij PE: Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet Infect Dis 2004; 4: 349–357.
12. Pfeiffer CD, Fine JP, Safdar N: Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis 2006; 42: 1417–1427.
13. Aquino VR, Goldani LZ, Pasqualotto AC: Update on the contribution of galactomannan for the diagnosis of invasive aspergillosis. Mycopathologia 2007; 163: 191–202.
14. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, et al. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of br onchoalveolar D-mannitol and serum galactomannan as markers of infection. J Infect Dis 1994; 169: 356–368.
15. Odabasi Z, Mattiuzzi G, Estey E, Kantarjian H, Saeki F, Ridge RJ, et al. β-D-Glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. Clin Infect Dis 2004; 39: 199–205.
16. Pazos C, Ponto J, Del Palacio A: Contribution of (1 → 3)-β-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. J Clin Microbiol 2005; 43: 299–305.
17. Tasaka S, Hasegawa N, Kobayashi S, Yamada W, Nishimura T, et al. Serum indicators for the diagnosis of Pneumocystis pneumonia. Chest 2007; 131: 1173–1180.
18. Kato A, Takita T, Furuhashi M, Takahashi T, Maruyama Y, Hishida A: Elevation of blood (1 → 3)-beta-D-glucan concentrations in hemodialysis patients. Nephron 2001; 89: 15–19.
19. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 2002; 347: 408–415.
20. Walsh TJ, Pappas P, Winston DJ, Lazarus HM, Petersen F, et al. Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 2002; 346: 225–234.

doi: 10.1111/j.1346-8138.2009.00624.x

ORIGINAL ARTICLE

Comparative study of direct polymerase chain reaction, microscopic examination and culture-based morphological methods for detection and identification of dermatophytes in nail and skin samples

Takao UCHIDA,[1,2] Koichi MAKIMURA,[2,3] Katsuhito ISHIHARA,[2,4] Hideaki GOTO,[2] Yoshito TAJIRI,[2] Mariko OKUMA,[2] Ryuichi FUJISAKI,[2] Katsuhisa UCHIDA,[2] Sigeru ABE,[2] Masafumi IIJIMA[1]

[1]*Department of Dermatology, School of Medicine, Showa University, Shinagawa-ku,* [2]*Institute of Medical Mycology, Graduate School of Medical Science, Teikyo University, Hachioji,* [3]*Genome Research Center, Graduate School of Medical Science, Teikyo University, Hachioji, and* [4]*Yamato Scientific, Aiko-gun, Tokyo, Japan*

ABSTRACT

The positive rates of dermatophytes isolated and identified by conventional methods are rather low. Moreover, clinical isolates sometimes show atypical morphology, and in such cases microscopic methods are not applicable for identification. The present study was performed to assess the utility of specific polymerase chain reaction (PCR)-based methods for *Trichophyton rubrum* and *Trichophyton mentagrophytes* as diagnostic tools for dermatophytoses. Both conventional morphological identification and specific PCR methods based on the nuclear ribosomal internal transcribed spacer (ITS)1 DNA sequence were performed to identify dermatophyte species from clinical specimens of patients who visited Kawasaki Social Insurance Hospital between 16 May and 17 August 2005. Specific PCR methods were also directly applied to clinical specimens, and the results of the two methods were compared. The clinical samples examined consisted of 126 skin scale specimens and 80 nail specimens. The positive rates of culture isolation from clinical specimens were 67% and 33% for skin scale and nail specimens, respectively. In contrast, PCR analysis yielded a positive rate of 100% for clinical isolates from both skin scales and nails, and rates of 95% and 99% were obtained by direct application to clinical specimens. The results of the present study indicated that specific PCR is highly advantageous as a diagnostic tool for detection and identification of dermatophytes on direct application to skin scale or nail specimens.

Key words: dermatophytes, identification, nuclear ribosomal internal transcribed spacer, nested polymerase chain reaction, polymerase chain reaction.

INTRODUCTION

Dermatophytosis is one of the most prevalent human infectious diseases, the principal etiological agents of which are *Trichophyton rubrum* (71.9%) and *Trichophyton mentagrophytes* (27.1%).[1,2] Together, these two species account for 99% of cases of dermatophytosis identified in Japan [2]. Tinea pedis and tinea unguium were found in 1474 and 818 of 8804 patients in Japan (16.7% and 9.3%), respectively, whose complaint on the first visit was "other than athlete's foot".[3] Tinea pedis was the most frequent type of dermatophytosis (60.2%) followed by tinea unguium (26.6%).[2]

Standard methods for identification of dermatophytes are based on the morphology, biochemical features, or mating ability of the isolates.[1]

However, due to the low positive rate[4–7] of isolating the pathogens and because clinical isolates sometimes

Correspondence: Koichi Makimura, M.D., Institute of Medical Mycology, Graduate School of Medical Science, Teikyo University, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan. Email: makimura@main.teikyo-u.ac.jp
Received 7 May 2008; accepted 24 December 2008.

© 2009 Japanese Dermatological Association

show atypical morphology, a new more reliable tool is required for detection and identification of the infectious agents.

Recently, molecular biological techniques have been used successfully to identify dermatophytes both precisely and rapidly.[8–11] Several methods for identifying dermatophyte species have been reported to date, including polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) targeting the DNA topoisomerase II gene,[12] RFLP analysis of PCR-amplified ribosomal DNA (rDNA) including internal transcribed spacers,[13] random amplification of polymorphic DNA (RAPD) analysis with random primers,[14] nested PCR based on DNA sequences of the nuclear ribosomal internal transcribed spacer (ITS)1 region,[15] and direct sequencing of PCR products.[16] Moreover, there have been a few reports of comparative studies between morphological and PCR analysis applied directly to nail specimens, including PCR-RFLP targeting the ITS region,[17] and T1 PCR,[18] al-though those studies have not been applied to skin scales.

The present study was performed to compare the results obtained by both conventional and nested PCR-based methods. For the nested PCR-based method, we used primer pairs specific for *T. rubrum* and *T. mentagrophytes* because these two species account for 99% of dermatophytes identified in Japan.[2]

METHODS

Clinical specimens

A total of 206 specimens from 133 patients positive for fungal elements by direct microscopy who visited the dermatological section of Kawasaki Social Insurance Hospital (Kanagawa, Japan) between 16 May and 17 August 2005 were examined in this study. Clinical specimens consisted of 126 skin scales (114 tinea pedis, one tinea manus, nine tinea corporis and two tinea cruris) and 80 nail specimens.

Morphological identification

Primary culture was performed by inoculation of clinical specimens onto Sabouraud's dextrose agar (SDA) medium supplemented with gentamicin sulfate, tetracycline hydrochloride, phenol red (Dermakit; Kyoritsu Seiyaku, Tokyo, Japan). To obtain pure cultures, primary colonies were passaged on SDA supplemented with chloramphenicol (BBL; Becton Dickinson, Franklin Lakes, NJ, USA), and were incubated at 27°C for up to 2 months. Identification by morphological methods was performed based on the typical macroscopic and microscopic characteristics. Species other than dermatophytes were not identified in this study.

Preparation of DNA from fungal cells

DNA was prepared from the isolates by the rapid method described by Makimura *et al.*[19] Briefly, small amounts of mycelia grown on SDA were placed in lysis buffer (200 mmol/L Tris-HCl, pH 7.5, with 25 mmol/L ethylene diamine tetra acetate (EDTA), 0.5% w/v sodium dodecylsulfate (SDS), and 250 mmol/L NaCl), and crushed with a conical grinder. The samples were then incubated at 100°C for 20 min and mixed with 150 µL of 3.0 mol sodium acetate, kept at –20°C for 10 min, and then centrifuged at 12 000 *g* for 10 min. The supernatants were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and subsequently extracted once with chloroform. DNA was precipitated with an equal volume of isopropanol, washed with 150 µL of 70% ethanol, dried and suspended in 50 µL of ultrapure water (Milli-Q Synthesis A10; Millipore, Bedford, MA, USA). Aliquots of 2 µL of the resultant solutions were used as templates for the first PCR. The total time required to prepare the DNA was 80 min.

Preparation of DNA from clinical specimens

Skin scales or nail specimens, the same amount of material required by culture method, were kept at –150°C, crushed with a mechanical crusher (Multi-Beads Shocker; Yasui Kikai, Osaka, Japan), and placed in lysis buffer (200 mmol/L Tris-HCl, pH 7.5, with 25 mmol/L EDTA, 0.5% w/v SDS, and 250 mmol/L NaCl). The samples were then incubated at 100°C for 10 min and mixed with 150 µL of 3.0 M sodium acetate, kept at –20°C for 10 min, and then centrifuged at 12 000 *g* for 10 min. The supernatants were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and subsequently extracted once with chloroform. DNA was precipitated with an equal volume of isopropanol, washed with 150 µL of 70% ethanol, dried and suspended in 50 µL of ultrapure water (Milli-Q Synthesis A10; Millipore). Aliquots of 2 µL of the resultant solutions were used as templates for the first PCR. The total time required to prepare the DNA was 80 min.

© 2009 Japanese Dermatological Association

Specific oligonucleotides

The universal ITS1-specific primer pairs were reported by Makimura *et al.*[16] *T. rubrum*-specific primers (RubF1, 5′-CCATTCTTGTCTACCTCACC-3′ and RubR1, 5′-CTCAGACTGACAGCTCTTCA-3′), *Arthroderma vanbreuseghemii*-specific cluster, including *T. mentagrophytes*,[19] primers (VanF1, 5′-ACGATAGGGCCAAACGTCCG-3′ and VanR1, 5′-TCCAGCGTTGAGCCACTAAAG-3′) were newly designed according to the respective species-specific ITS1 base sequences.[16]

PCR

Each PCR assay mixture contained 5 μL of 10 × reaction buffer (Amersham Biosciences, Piscataway, NJ, USA), 50 μmol each of dATP, dCTP, dGTP and dTTP (Amersham Biosciences), 1.25 U of Taq polymerase (Amersham Biosciences), 15 pmol of each primer and the DNA template solution. Ultrapure water was added to bring the volume to 50 μL.

First PCR

Mixtures containing DNA extracted from fungal strains or clinical specimens were heated to 94°C for 4 min and PCR was performed for 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 90 s, with a final extension step of 10 min at 72°C.[20]

Nested PCR

Reaction mixtures containing 1/100 diluted first PCR products were heated to 94°C for 4 min and PCR was performed for 30 cycles of 94°C for 1 min, 60°C for 15 s and 72°C for 15 s, with a final extension step of 10 min at 72°C.[21]

Detection of PCR products

Aliquots of 5 μL of amplified products were electrophoresed through 1.2% agarose gels in Tris-borate-EDTA (TBE) buffer and visualized by staining with ethidium bromide under ultraviolet irradiation.

RESULTS

Specificity of *T. mentagrophytes* and *T. rubrum*-specific PCR

The specificities of *T. mentagrophytes* and *T. rubrum*-specific PCR for standard strains (Table 1) were examined using the primers vanF1 and vanR1, and rubF1 and rubR1, respectively. A product of approximately 0.15 kb was amplified from *A. vanbreuseghemii*, a teleomorph of the *T. mentagrophytes* complex, and *T. mentagrophytes* var. *interdigitale* by PCR with vanF1 and vanR1, but not from other fungi examined. Another product of approximately 0.15 kb was amplified from *T. rubrum* by PCR with the primers rubF1 and rubR1, but not from other fungi examined. No amplification product was observed in controls without a template (data not shown).

Identification of pathogens from clinical isolates by culture-based morphological method

The following features of the colonies and microscopic morphology were taken to indicate *T. rubrum*: (i) downy white or bright red color with a flat or slightly raised center, otherwise white, cream or pale red color with a powdery to suede-like surface with a radial ditch in the colony obverse; (ii) wine-red or dark brown colony reverse pigmentation; and (iii) clavate teardrop-shaped microconidia (measuring 2–3 × 3–5 μm) lateral to the hyphae on microscopic morphology. On the other hand, those isolates showing the following features were classified as *T. mentagrophytes*: (i) cream, buff-yellow or brown color with various powdery, granulated, velvet or cotton texture, and a flat or upraised colony surface; (ii) usually cream, brown or reddish brown, sometimes yellow or light yellow colony reverse pigment; and (iii) globose microconidia in grapelike clusters, and spiral hyphae on microscopic morphology. Cases that did not show these typical morphological characteristics were classified as "unidentified". One of the unidentified nail samples was *T. mentagrophytes* based on PCR of clinical isolates, and the other unidentified skin and nail samples were *T. rubrum* based on PCR of clinical isolates.

Identification of pathogens from clinical isolates cultured on SDA by nested PCR

Figure 1(a) shows a typical electropherogram of first PCR products from isolates amplified with universal ITS1 primers (18SF1 and 58SR1). Figure 1(b,c) show nested PCR products amplified with *T. rubrum*-specific primers (rubF1 and rubR1) and *A. vanbreuseghemii*-specific primers (vanF1 and vanR1), respectively. All DNA prepared from fungal cells were amplified successfully with the universal ITS1 primers. The sample DNA were amplified by both subsequent nested PCR using *T. rubrum*-specific primers and

© 2009 Japanese Dermatological Association

Table 1. Specificity of the primer-system

Species	Strain	Primer-pair		
		ITS1[†]	VAN[‡]	RUB[§]
Arthroderma benhamiae	VUT77011	+	–	–
	VUT77012	+	–	ND
Arthroderma simii	VUT77009	+	–	–
	VUT77010	+	–	ND
Arthroderma vanbreuseghemii	VUT77007	+	+	–
	VUT77008	+	+	ND
Trichophyton mentagrophytes var. *interdigitale*	TIMM3258.	–	+	–
Trichophyton tonsulans	TIMM1254	+	–	–
Trichophyton rubrum	TIMM3276	+	–	+
	TIMM3223	+	ND	+
Trichophyton verrucosum	CBS563.50	+	–	–
Trichophyton terrestre	TIMM3394	+	–	–
Microsporum gypseum	TIMM3397	+	–	+
Microsporum cookiei	TIMM3398	+	–	–
Microsporum canis	TIMM1502	+	–	–
	TIMM0765	+	–	–
Microsporum audounii	TIMM0757	+	–	–
Epidermophyton floccosum	TIMM0431	+	–	–
Exophiala dermatitidis	TIMM0439	+	–	–
Fonsecaea pedrosoi	TIMM0482	+	–	–
Aspergillus fumigatus	TIMM1776	+	–	–
Aspergillus flavus	TIMM0057	+	–	–
Aspergillus nigar	TIMM0113	+	–	–
Penicillium marneffei	IFM41707	+	–	–
Candida albicans	ATCC90029	+	–	–
Candida glabrata	TIMM1062	+	–	–
Cryptococcus neoformans	TIMM1855	+	–	–
Mucor circinelloides	TIMM1324	+	–	–
Rhizopus oryzae	TIMM1326	+	–	–

[†]ITS1, internal transcribed spacer 1 (ITS1) universal primer. [‡]VAN, *A. vanbreuseghemii*-specific cluster, including *T. mentagrophytes*, primer. [§]RUB, *T. rubrum*-specific primer.

subsequent nested PCR using *A. vanbreuseghemii*-specific primers. The PCR results indicated the coexistence of *T. rubrum* and *T. mentagrophytes* in cultures from sample number 6.

Identification of pathogens from clinical specimens by nested PCR

Figure 2(a) shows a typical electropherogram of first PCR products from clinical specimens amplified using the universal ITS1 primers. Figure 2(b,c) show nested PCR products amplified with *T. rubrum*-specific primers and *A. vanbreuseghemii*-specific primers, respectively. Not all DNA prepared from clinical specimens were amplified with universal ITS1 primers in first PCR. However, DNA were amplified successfully by subsequent nested PCR using *T. rubrum*-specific primers and/or *A. vanbreuseghemii*-specific primers. These results also indicated the coexistence of *T. rubrum* and *T. mentagrophytes* based on direct PCR of clinical specimens.

Comparison between identification based on morphology, and PCR from cultures and clinical specimens

The results of culture- and PCR-based analyses of clinical isolates cultured on SDA, and PCR analysis performed directly on clinical specimens are summarized in Table 2. Culture-based analysis from skin scales and nail specimens gave positive results in 84 (67%) and 26 subjects (33%), respectively. PCR analysis of clinical isolates gave positive results in 88 (100%) and 28 subjects (100%) from skin scales and nail specimens, respectively. Direct PCR analysis of skin scales and nail specimens gave positive results for 120 (95%) and 79 subjects (99%), respectively. The incidence of mixed infection with *T. rubrum* and

© 2009 Japanese Dermatological Association

Table 2. Comparison between identification based on morphology, polymerase chain reaction (PCR) from cultures and PCR from specimens

Method		Type	Total no.	TR/TM†	Positive TR‡(%)	TM§(%)	TR + TM¶(%)	Unidentified#(%)	Negative(%)
Culture	Morphology	Skin	126	1.02	42 (33)	41 (33)	1 (1)	4 (3)	38 (30)
		Nail	80	5.5	22 (28)	4 (5)	0 (0)	2 (3)	52 (65)
	PCR	Skin	88	1.04	42 (48)	40 (45)	6 (7)	0 (0)	0 (0)
		Nail	28	3.29	21 (75)	5 (18)	2 (7)	0 (0)	0 (0)
PCR from specimen		Skin	126	1.12	60 (48)	53 (42)	7 (6)	0 (0)	6 (5)
		Nail	80	5.54	66 (84)	7 (9)	6 (8)	0 (0)	1 (1)

†TR/TM ratio indicated the numerical value that *Trichophyton rubrum*-positive subjects divided by *Trichophyton mentagrophytes*-positive subjects. ‡TR, *T. rubrum*. §TM, *T. mentagrophytes*. ¶TR + TM, mixed infection of *T. rubrum* and *T. mentagrophytes*. #Unidentified represent isolates that identification by morphology was difficult.

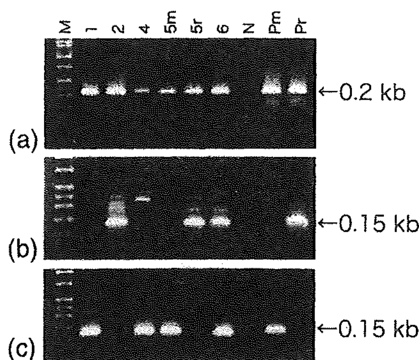


Figure 1. Identification of clinical isolates cultured on Sabouraud's dextrose agar (SDA) by first and nested polymerase chain reaction (PCR). Amplification profiles of the PCR products generated from the clinical isolates cultured on SDA; electropherograms of PCR using internal transcribed spacer (ITS)1 primers (a), *Trichophyton rubrum*-specific primers (b), *Arthroderma vanbreuseghemii*-specific primer (c). The numbers at the top of each lane in (a) indicate the sample numbers. M, DNA molecular weight marker IX (Roche, Mannheim, Germany); Pm, *Trichophyton mentagrophytes* (positive control); Pr, *T. rubrum* (positive control); N,TE8.0 (2 mol/L Tris-Hcl, pH 8.0, with 500 m mol/l ethylene diamine tetra acetate) (negative control). Both *T. rubrum* and *T. mentagrophytes* were cultured from the clinical specimen sample number 5. Therefore, 5 m indicates the *T. mentagrophytes* isolate and 5 r indicates the *T. rubrum* isolate from clinical specimen sample number 5. All DNA prepared from fungal cells were successfully amplified with the universal ITS1 primers. DNA from sample numbers 2, 5 r and 6 were amplified by subsequent nested PCR using *T. rubrum*-specific primers, and DNA from sample numbers 1, 4, 5 m and 6 were amplified by subsequent nested PCR using *A. vanbreuseghemii*-specific primers. Sample number 6 showed the coexistence of *T. rubrum* and *T. mentagrophytes* based on PCR from culture isolates.

T. mentagrophytes was higher than that reported in a previous study by Kasai[7] using culture-based morphological methods. Moreover, there was disagreement among the three methods in seven of 88 skin scales samples (8%) and four of 28 nail samples (14%).

With universal ITS1 primers only, first PCR of clinical isolates yielded positive results for all subjects from skin scales and nail specimens. However, PCR of clinical specimens gave positive results in 37 (29%) and 58 subjects (73%) from skin scales and nail specimens, respectively. The nail specimens showed a high positive ratio in the results of PCR analysis with the universal ITS1 primers in comparison with skin scale specimens ($P < 0.01$, χ^2-test).

DISCUSSION

We compared the results of morphological analysis with those of PCR-based analyses of clinical isolates cultured in SDA and of clinical specimens obtained from dermatophytosis patients. In comparison with the results of culture-based methods, PCR-based analyses of clinical specimens showed excellent positive results when applied to both skin scale and nail samples. PCR-based methods enable the accurate identification of the species in clinical dermatophytosis specimens within 1 day, even in cases in which no isolates are obtained by culture.

While there were disagreements among the three methods in individual samples, this was thought to be due to the coexistence of *T. rubrum* and *T. mentagrophytes* in some samples. Laboratory interpretation for negative reaction of each method was thought

© 2009 Japanese Dermatological Association

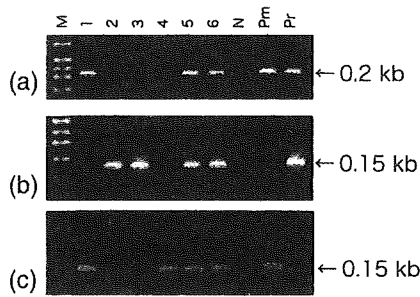


Figure 2. Identification of pathogens from clinical specimens by first and nested polymerase chain reaction (PCR). Amplification profiles of the PCR products generated from the clinical specimens: electropherograms of PCR using internal transcribed spacer (ITS)1 primers (a), *Trichophyton rubrum*-specific primers (b), and *Arthroderma vanbreuseghemii*-specific primers (c). The numbers at the top of each lane in (a) indicate the sample numbers. M, DNA molecular weight marker IX (Roche); Pm, *Trichophyton mentagrophytes* (positive control); Pr, *T. rubrum* (positive control); N,TE8.0 (2 mol/L Tris-Hcl, pH 8.0, with 500 m mol/l ethylene diamine tetra acetate) (negative control). Although sample number 3 was a negative based on morphological analysis, it was a positive based on PCR analysis. DNA from sample numbers 1, 5, 6, Pm and Pr were amplified successfully with universal ITS1 primers. Then, DNA from sample numbers 2, 3, 5 and 6 were amplified by subsequent nested PCR using *T. rubrum*-specific primers, and those from sample numbers 1, 4, 5 and 6 were amplified by subsequent nested PCR using *A. vanbreuseghemii*-specific primers. Therefore, sample numbers 5 and 6 showed the coexistence of *T. rubrum* and *T. mentagrophytes* based on PCR from clinical specimens.

to be sampled in an effete (dead filaments) area, or PCR inhibitors were present.

Single or mixed non-dermatophyte infection is thought to be a cause of onychomycosis.[22] However, non-dermatophytes were excluded in this study, because true non-dermatophytic onychomycosis accounts for only a relatively small proportion of all cases of onychomycosis.

In comparison with the specimens from skin scales, those from the nails showed a high positive ratio in PCR analysis of clinical specimens using the ITS1 universal primers. We postulated that this was because the nails could be crushed more easily than the skin scales with the mechanical crusher due to their greater hardness, and this would facilitate preparation of DNA.

In conclusion, nested PCR of clinical specimens is a rapid and accurate method of identifying species of dermatophytes. The application of nested PCR to dermatophytes should be particularly useful because the positive rate of isolation and identification of dermatophytes by conventional methods is rather low, and clinical isolates sometimes show atypical morphology that prevents identification using microscopic methods.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (K. M.).

REFERENCES

1 Rippon JW. Dermatophytosis and dermatomycosis. In: Rippon JW (ed). *Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes*. W.B. Saunders, Philadelphia, 1988; 169–275.
2 Nishimoto K. An epidemiological survey of dermatomycoses in Japan, 2002. *Nippon Ishinkin Gakkai Zasshi* 2006; **47**: 103–111. (in Jpn)
3 Watanabe S, Nishimoto K, Asanuma H *et al*. [An Epidemiological Study to Assess the Prevalence of Tinea Pedis et Unguium in Japan.] *Nippon Hifuka Gakkai Zasshi*. 2001; **111**: 2101–2112. (In Japanese.)
4 Ogasawara Y, Hiruma M, Muto M, Ogawa H. Clinical and mycological study of occult tinea pedis and tinea unguium in dermatological patients from Tokyo. *Mycoses* 2003; **46**: 114–119.
5 Falahati M, Akhlaghi L, Lari AR, Alaghehbandan R. Epidemiology of dermatophytoses in an area south of Tehran, Iran. *Mycopathologia* 2003; **156**: 279–287.
6 Aste N, Pau M, Aste N, Biggio P. Tinea pedis observed in Cagliari, Italy, between 1996 and 2000. *Mycoses* 2003; **46**: 38–41.
7 Kasai T. [Statistical study of dermatomycosis for 30 years (1968–1997) in Sendai National Hospital.] *Nippon Ishinkin Gakkai Zasshi* 2004; **45**: 149–163. (In Japanese.)
8 Liu D, Pearce L, Lilley G, Coloe S, Baird R, Pedersen J. PCR identification of dermatophyte fungi *Trichophyton rubrum*, *T. soudanense* and *T. gourvilii*. *J Med Microbiol* 2002; **51**: 117–122.
9 Gräser Y, el Fari M, Presber W, Sterry W, Tietz HJ. Identification of common dermatophytes (*Trichophyton*, *Microsporum*, *Epidermophyton*) using polymerase chain reactions. *Br J Dermatol* 1998; **138**: 576–582.
10 Summerbell RC, Haugland RA, Li A, Gupta AK. rRNA gene internal transcribed spacer 1 and 2 sequences of asexual, anthropophilic dermatophytes related to *Trichophyton rubrum*. *J Clin Microbiol* 1999; **37**: 4005–4011.
11 Liu D, Coloe S, Pedersen J, Baird R. Use of arbitrarily primed polymerase chain reaction to differentiate

© 2009 Japanese Dermatological Association

Trichophyton dermatophytes. *FEMS Microbiol Lett* 1996; **136**: 147–150.

12 Kamiya A, Kikuchi A, Tomita Y, Kanbe T. PCR and PCR-RFLP techniques targeting the DNA topoisomerase II gene for rapid clinical diagnosis of the etiologic agent of dermatophytosis. *J Dermatol Sci* 2004; **34**: 35–48.

13 Jackson CJ, Barton RC, Evans EG. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. *J Clin Microbiol* 1999; **37**: 931–936.

14 Mochizuki T, Sugie N, Uehara M. Random amplification of polymorphic DNA is useful for the differentiation of several anthropophilic dermatophytes. *Mycoses* 1997; **40**: 405–409.

15 Nagao K, Sugita T, Ouchi T, Nishikawa T. Identification of *Trichophyton rubrum* by nested PCR analysis from paraffin embedded specimen in trichophytia profunda acuta of the glabrous skin. *Nippon Ishinkin Gakkai Zasshi* 2005; **46**: 129–132.

16 Makimura K, Tamura Y, Mochizuki T *et al*. Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. *J Clin Microbiol* 1999; **37**: 920–924.

17 Yoshimura R, Ito Y, Morishita N *et al*. Comparative study between culture and PCR-RFLP analysis on identification of the causative agent of tinea unguium. *Nippon Ishinkin Gakkai Zasshi* 2006; **47**: 11–14.

18 Kardjeva V, Summerbell R, Kantardjiev T *et al*. Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. *J Clin Microbiol* 2006; **44**: 1419–1427.

19 Makimura K. Species identification system for dermatophytes based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1. *Nippon Ishinkin Gakkai Zasshi*. 2001; **42**: 61–67.

20 Makimura K, Murayama SY, Yamaguchi H. Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Clin Microbiol* 1994; **40**: 358–364.

21 Makimura K, Tamura Y, Murakami A *et al*. Cluster analysis of human and animal pathogenic *Microsporum* species and their teleomorphic states, *Arthroderma* species, based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1. *Microbiol Immunol* 2001; **45**: 209–216.

22 Summerbell RC, Kane J, Krajden S. Onychomycosis, tinea pedis and tinea manuum caused by non-dermatophytic filamentous fungi. *Mycoses* 1989; **32**: 609–619.

© 2009 Japanese Dermatological Association

Jpn. J. Infect. Dis., 62, 164-167, 2009

Method

Direct Colony PCR of Several Medically Important Fungi Using Ampdirect® Plus

Mohamed M. AlShahni[1,2], Koichi Makimura[1]*, Tsuyoshi Yamada[1], Kazuo Satoh[1], Yumiko Ishihara[1], Kosuke Takatori[2], and Takuo Sawada[2]

[1]*Teikyo University Institute of Medical Mycology, Tokyo 192-0395, and*
[2]*Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan*

(Received June 26, 2008. Accepted December 17, 2008)

SUMMARY: Direct colony polymerase chain reaction (DCPCR) is a useful molecular biological technique for application in the field of mycology. In this study, all of the 63 fungal strains examined, including those of the genera *Candida* and *Aspergillus*, were amenable to DNA amplification using an Ampdirect® Plus kit, which allows direct PCR amplification with no requirement for DNA extraction, following 1 h of rapid fungal lysis. Moreover, we compared DCPCR of 35 strains, representing 20 species, using Ampdirect PCR and standard PCR with no lysis buffer. Thirty-four of these strains (97.14%) yielded positive results on Ampdirect PCR, while only 11 (including *Aspergillus fumigatus* TIMM 1776) of the 35 strains (31.43%) showed PCR products when standard PCR reagents were used. Ampdirect DCPCR was also applicable to DNA amplification for spore and hyphal cells. This approach reduces DNA template preparation time before PCR from fungal colonies, and also reduces the cost of PCR.

INTRODUCTION

The incidence of mycosis has increased markedly over the last several years (1,2). Polymerase chain reaction (PCR) has been shown to be a useful method in the field of mycology for the diagnosis of fungal infections as well as for the identification and taxonomy, etc. Such research requires a number of laborious steps, including preparation of a DNA template by extraction of DNA from fungi, for which a number of methods have been reported (3-5). However, direct colony polymerase chain reaction (DCPCR) of fungal cultures would be an ideal solution when it is applicable. Unlike molds, DCPCR of yeasts has been reported frequently (6-8). In contrast, only a few reports have described the possibility of direct PCR from hyphae of a limited number of mold species (7,9).

To reduce PCR cost, labor, and time, to avoid the risk of contamination during the DNA extraction process (10), and to obviate the use of extraction materials, we proposed Ampdirect DCPCR to amplify the internal transcribed spacer (ITS) region of fungal rDNA of numerous fungi with or without a lysis step. This approach involves the use of an Ampdirect® Plus kit, which allows direct PCR amplification with no requirement for DNA extraction (Shimadzu, Kyoto, Japan). The manufacturer's data sheet recommends use of this kit for direct PCR after lysis in tissues and fluids from animals or plants, and outlines its tolerance to PCR inhibitors. In addition, Ampdirect® Plus was successfully used to genotype *Aspergillus oryzae* following DNA extraction on FTA cards (11) but was not applied to mycelia directly. The Ampdirect PCR protocol involves digestion of clinical samples using a special buffer, Ampdirect lysis buffer (ALB), which lyses fungal cell walls at 55°C for more than 1 h followed by boiling for 5 min. The resulting lysate is used as the DNA template for PCR in a total volume of 20 μl in accordance with the manufacturer's protocol.

In the present study, we demonstrated the applicability of DCPCR in numerous mold and yeast strains. This technique will facilitate PCR for both clinical and experimental purposes using Ampdirect® Plus.

MATERIALS AND METHODS

Strains and media: Most of the 63 strains examined in this study, all of which were from the Teikyo University Institute of Medical Mycology (TIMM) culture collection, were of medical importance. They consisted of 27 yeast strains belonging to 15 species from 7 genera (*Candida*, *n* = 8; *Clavispora*, *n* = 1; *Pichia*, *n* = 1; *Saccharomyces*, *n* = 2; *Schizosaccharomyces*, *n* = 1; *Filobasidiella*, *n* = 3; and *Trichosporon*, *n* = 1) and 36 mold strains from 20 species belonging to 4 genera (*Aspergillus*, *n* = 15; *Eurotium*, *n* = 3; *Penicillium*, *n* = 1; and *Fusarium*, *n* = 1) (Tables 1 and 2). Yeast and mold strains were cultured on Sabouraud dextrose agar (SDA) plates at 25°C for 7-10 days. Four strains–*Aspergillus sclerotiorum* JCM 1962, *A. unguis* JCM 2256, *Eurotium amstelodami* JCM 1565, and *Penicillium expansum* TIMM 1293–were used to perform direct PCR for hyphae and spores. They were grown in potato dextrose broth (PDB) at 25°C for 48 h in order to extract hyphae.

Lysis buffers and DNA template preparation: The ALB and three additional lysis buffers, derived from ALB by excluding one or more of its components, were used. The buffers were: (i) ALB (20 mM Tris-HCl, pH 8.0, 5 mM ethylenediaminetetraacetic acid (EDTA), 400 mM NaCl, 0.3% sodium dodecyl sulfate (SDS), and 200 μg/ml proteinase K); (ii) proteinase K-free ALB; (iii) SDS-free ALB; and (iv) proteinase K- and SDS-free ALB. These four buffers were stored at –20°C.

DNA templates were prepared by suspending portions of fungal cultures in 25 μl of ultrapure water, and aliquots of 5 μl of the suspensions were added to 70 μl of each lysis

*Corresponding author: Mailing address: Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192-0395 Japan. Tel: +81-426-78-3256, Fax: +81-426-74-9190, E-mail: makimura@main.teikyo-u.ac.jp

Table 1. Mold strains examined by Ampdirect PCR protocol

Strain	Strain
Aspergillus fumigatus[1)]	*Aspergillus niger*[2)]
Aspergillus terreus var. *terreus* JCM 10257	*Aspergillus candidus* JCM 10250
Aspergillus nidulans var. *nidulans* JCM 2728	*Aspergillus clavatus* JCM 10080
Aspergillus versicolor JCM 10258	*Aspergillus conicus* JCM 1725
Aspergillus flavus JCM 2061	*Aspergillus unguis* JCM 2256
Aspergillus sclerotiorum JCM 1962	*Aspergillus sydowii* JCM 2722
Aspergillus tamarii JCM 2259	*Aspergillus penicillioides* JCM 10256
Aspergillus ochraceus JCM 10255	*Penicillium expansum* TIMM 1293
Eurotium amstelodami JCM 1565	*Eurotium chevalieri* JCM 1568
Eurotium herbariorum JCM 1575	*Fusarium moniliforme* TIMM 1294

[1)]: Thirteen strains of *A. fumigatus* were examined; *A. fumigatus* TIMM 2922, TIMM 0108, TIMM 2916, TIMM 2904, TIMM 2905, TIMM 2906, TIMM 2907, TIMM 2908, TIMM 2909, TIMM 2914, TIMM 2913, TIMM 1776, and *A. fumigatus* var. *fumigatus* JCM 10253.
[2)]: Five strains of *A. niger* were examined; *A. niger* JCM 10254, TIMM 0114, TIMM 0115, TIMM 2911, and TIMM 2915.

Table 2. Yeast strains examined by Ampdirect PCR protocol

Strain
Candida albicans ATCC 10231 and ATCC 90028
Candida fukushimaensis Lucy colony M 9/1
Candida guilliermondii TIMM 0257 and TIMM 3400
Clavispora lusitaniae TIMM 1667 and TIMM 2941
Candida dubliniensis CBS 7987, CBS 7988 and CBS 2747
Candida glabrata ATCC 90030 and CBS 138
Candida parapsilosis ATCC 90018 and ATCC 22019
Candida tropicalis ATCC 750 and Lucy colony S 9/1
Candida krusei ATCC 6258 and TIMM 3404
Filobasidiella neoformans var. *neoformans* ATCC 90113, CN03010902 and INOKICHI
Pichia anomala TIMM 3420 and TIMM 3826
Saccharomyces cerevisiae ATCC 9763
Saccharomyces pastorianus ATCC 2366
Schizosaccharomyces pombe L972h
Trichosporon asahii JCM 2466

buffer in microtubes and mixed thoroughly. Subsequently, they were incubated at 55°C for 1 h followed by 5 min of boiling when ALB or SDS-free ALB was used. The resulting lysates were used as DNA template sources. In case of Ampdirect DCPCR, where no lysing buffer was employed, the DNA template was obtained directly from the previous fungal suspension in ultrapure water.

Hyphae and spore preparation: Hyphae from the broth cultures of the four strains were drawn out through 40 μm filters, washed twice with 2 ml of ultrapure water, and centrifuged at 3,000 rpm for 4 min. Portions of about 15 mg of the resulting hyphae were suspended in 25 μl ultrapure water and used as the DNA template.

Spores were prepared by gently washing the surfaces of 5- to 7-day-old plate cultures with 1.5 ml of 0.1% Tween 20, and the suspension was centrifuged at 4,800 $\times$ *g* for 10 min. After aspiration of the supernatant, spores were washed twice with 500 μl of ultrapure water. Finally, the efficiency of spore extraction was confirmed by microscopic examination. A concentration of 1×10^3 - 1×10^4 cells per PCR reaction was used in our experiments as a DNA template (12).

PCR setup, conditions, and product screening: A pair of primers were used for PCR: ITS1 primer (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) (13). Ampdirect PCR was performed in a total reaction volume of 20 μl containing 2$\times$ Ampdirect® Plus, 0.5 U of NovaTaq™ Hot Start DNA Polymerase (Novagen, Nottingham, UK), 12 pmol ITS1, 12 pmol ITS4, and 0.5 μl of DNA template. Standard PCR was performed in a total reaction volume of 20 μl containing 10$\times$ buffer (GE Healthcare Ltd., Buckinghamshire, UK), 0.5 U Taq DNA Polymerase (GE Healthcare), 4 mM dNTPs, 12 pmol ITS1, 12 pmol ITS4, and 0.5 μl of DNA template. Conditions for both Ampdirect and standard PCR were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. In all PCRs, we included a negative control and a positive control. Ultrapure water was used instead of DNA template in negative controls, while DNA extracted from *Cryptococcus neoformans* ATCC 90113 by the phenol-chloroform method was employed as positive controls.

Amplicons were separated by electrophoresis on 1.0% (w/v) agarose gels. The gels were stained with ethidium bromide and bands were visualized on a UV transilluminator.

RESULTS

The Ampdirect PCR protocol yielded PCR products from all 63 strains examined. Four of the 63 strains–*Filobasidiella neoformans* (*C. neoformans*) ATCC 90113, *Candida albicans* ATCC 10231, *Aspergillus fumigatus* TIMM 1776, and JCM 10253–were tested by the four different lysing buffers described above prior to Ampdirect PCR. Positive results were obtained with all four buffers (Fig. 1).

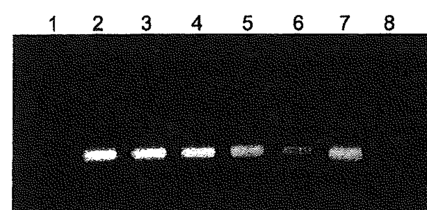


Fig. 1. Results for *Aspergillus fumigatus* JCM 10253 treated with different lysis buffers and amplified by Ampdirect® Plus. Lane 1, Marker. Lane 2, PCR after treatment with Ampdirect lysis buffer (ALB). Lane 3, PCR after treatment with sodium dodecyl sulfate (SDS)-free ALB. Lane 4, PCR after treatment with proteinase K-free ALB. Lane 5, PCR after treatment with SDS- and proteinase K-free ALB. Lane 6, direct colony PCR with no lysis step. Lane 7, positive control. Lane 8, negative control. Bands display with different intensities depending on the examined lysing buffers.

Table 3. Results of direct colony PCR (DCPCR) with Ampdirect and standard PCRs and without lysis steps as compared with the Ampdirect PCR protocol

Fungal species	DCPCR		Ampdirect PCR protocol[3]
	Ampdirect[1]	Standard[2]	
Aspergillus candidus JCM 10250	−	−	+
Aspergillus terreus var. *terreus* JCM 10257	+	−	+
Aspergillus nidulans var. *nidulans* JCM 2728	+	−	+
Aspergillus clavatus JCM 10080	+	−	+
Aspergillus flavus JCM 2061	+	−	+
Aspergillus tamarii JCM 2259	+	−	+
Aspergillus niger[4]	+	−	+
Aspergillus ochraceus JCM 10255	+	−	+
Aspergillus fumigatus[5]	+	−*	+
Aspergillus conicus JCM 1725	+	+	+
Aspergillus sydowii JCM 2722	+	+	+
Aspergillus versicolor JCM 10258	+	−	+
Aspergillus penicillioides JCM 10256	+	+	+
Aspergillus sclerotiorum JCM 1962	+	+	+
Aspergillus unguis JCM 2256	+	+	+
Eurotium chevalieri JCM 1568	+	+	+
Eurotium herbariorum JCM 1575	+	+	+
Eurotium amstelodami JCM 1565	+	+	+
Penicillium expansum TIMM 1293	+	+	+
Fusarium moniliforme TIMM 1294	+	+	+

1): Ampdirect DCPCR.
2): Standard DCPCR.
3): Ampdirect Lysis Buffer (ALB) prior to Ampdirect PCR.
4): Four strains of *A. niger* were examined; *A. niger* TIMM 0114, TIMM 0115, TIMM 2911, and TIMM 2915.
5): Thirteen strains of *A. fumigatus* were examined; *A. fumigatus* TIMM 2922, TIMM 0108, TIMM 2916, TIMM 2904, TIMM 2905, TIMM 2906, TIMM 2907, TIMM 2908, TIMM 2909, TIMM 2914, TIMM 2913, TIMM 1776, and *A. fumigatus* var. *fumigatus* JCM 10253.
*: None of the *A. fumigatus* strains showed any bands on standard PCR except *A. fumigatus* TIMM1776. (+): Generation of PCR products. (−): Absence of PCR products. The results were confirmed twice.

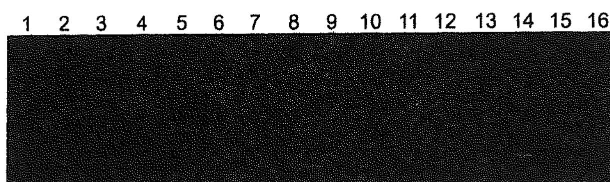


Fig. 2. Results of comparative direct colony PCR (DCPCR) using Ampdirect kit or standard PCR reagents. The left and right parts of the gel show Ampdirect DCPCR and DCPCR with standard PCR reagents, respectively. Lanes 1 and 9, Markers. Lanes 2 and 10, DCPCR of *Penicillium expansum* TIMM 1293. Lanes 3 and 11, DCPCR of *Aspergillus sclerotiorum* JCM 1962. Lanes 4 and 12, DCPCR of *A. niger* TIMM 0115. Lanes 5 and 13, DCPCR of *A. fumigatus* TIMM 0108. Lanes 6 and 14, DCPCR of *Fusarium moniliforme* TIMM 1294. Lanes 7 and 15, DCPCR of *A. tamarii* JCM 2259. Lanes 8 and 16, DCPCR of *A. nidulans* var. *nidulans* JCM 2728.

In light of the results described above, the DCPCR of 35 strains, representing 20 species, were compared using Ampdirect PCR and standard PCR with no lysing buffer (Fig. 2). Thirty-four of the 35 strains (97.14%) yielded positive results on Ampdirect PCR, while only 11 (including *A. fumigatus* TIMM 1776) of the 35 (31.43%) showed PCR products when standard PCR reagents were used (Table 3). On the other hand, Ampdirect DCPCR was also performed using the hyphae and spores of four filamentous fungi. Amplicons showed positive results in all four strains (Fig. 3).

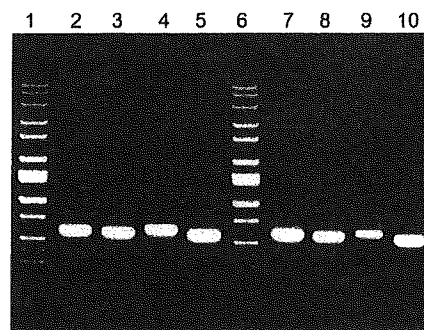


Fig. 3. Results of hyphae and spores Ampdirect direct colony PCR. The left and right parts of the gel show spores direct PCR and hyphae direct PCR, respectively. Lanes 1 and 6, Markers. Lanes 2 and 7, *Penicillium expansum* TIMM 1293. Lanes 3 and 8, *Aspergillus unguis* JCM 2256. Lanes 4 and 9, *A. sclerotiorum* JCM 1962. Lanes 5 and 10, *Eurotium amstelodami* JCM 1565.

DISCUSSION

We chose Ampdirect® Plus to develop a method for performing DCPCR in most fungal species, in part by virtue of its tolerance to PCR inhibitors. In the present study, we examined the applicability of the Ampdirect PCR protocol to a set of 63 fungal strains, and obtained positive results. The combination of ALB components and Ampdirect PCR following ALB lysis appeared to play a significant role in fungal wall lysing, which led to PCR product generation (14). This kit also seemed to be resistant to PCR inhibitors, such